Global **Malaria** Programme

# Test procedures for insecticide resistance monitoring in malaria vector mosquitoes

Second edition

World Health Organization

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This document was updated in June 2018. Please consult the website for any content updates (http://www.who.int/malaria)

WHO Library Cataloguing-in-Publication Data

Test procedures for insecticide resistance monitoring in malaria vector mosquitoes – 2nd ed.

1.Insecticide Resistance. 2.Malaria - prevention and control. 3.Insecticides. 4.Insect Control. I.World Health Organization.

ISBN 978 92 4 151157 5 (NLM classification: WA 240)

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# ACKNOWLEDGEMENTS

The following individuals contributed to the development of these test procedures and the Global Malaria Programme of the World Health Organization would like to acknowledge their valuable contribution: Birkinesh Ameneshewa, Bill Brogdon, Basil Brooke, Fabrice Chandre, Adanan Che Rus, Maureen Coetzee, Martin Donnelly, Josiane Etang, Christen Fornadel, Mary Anne Groepe, Pierre Guillet, Melinda Hadi, Jeffrey Hii, Zairi Jaal, Tessa Knox, Jan Kolaczinski, Abraham Mnzava, Martha Quinones, Hilary Ranson, Mark Rowland, Emmanuel Temu and Rajpal Yadav.

### Updates

Minor revisions were made to this edition in June 2018, mainly to: adapt Figure 3.1 to provide a more comprehensive overview; align with statistics provided in the latest *World malaria report*; and, include information and citations for other publications released recently.

## ABBREVIATIONS

| | |
|---|---|
| AI | active ingredient |
| CDC | US Centers for Disease Control and Prevention |
| DDT | dichlorodiphenyltrichloroethane |
| ELISA | enzyme-linked immunosorbent assay |
| GMP | WHO Global Malaria Programme |
| GPIRM | Global plan for insecticide resistance management in malaria vectors |
| IRS | indoor residual spraying |
| ITN | insecticide-treated mosquito net |
| *kdr* | knockdown resistance |
| LLIN | long-lasting insecticidal net |
| NMCP | national malaria control programme |
| PBO | piperonyl butoxide |
| PCR | polymerase chain reaction |
| *rdl* | resistance to dieldrin |
| WHO | World Health Organization |
| WHOPES | World Health Organization Pesticide Evaluation Scheme |

# GLOSSARY

| | |
|---|---|
| ***Ace-1*** | A target-site resistance gene for carbamate and organophosphate insecticides conferring insensitive acetylcholinesterase. The resistance is caused by a single mutation, G119S, of the *Ace-1* gene. |
| **cross-resistance** | Resistance to one insecticide by a mechanism that also confers resistance to insecticides of another class, even where the insect population or strain has not been selected by exposure to the latter. |
| **F1 progeny** | Generally means "first generation offspring", but in this context refers to the use of adults raised from the eggs of wild-caught female mosquitoes to obtain an age-standardized sample of the wild population for use in bioassay tests for resistance. |
| **insecticide combination** | Application of two or more insecticides with unrelated modes of actions within a house or a building (e.g. one insecticide class on the wall surfaces, and another class on nets used in the same household). Application of insecticide combinations differs from the use of insecticide mixtures in that, with a combination, an insect is likely but not guaranteed to come into contact with both insecticides. |
| **insecticide discriminating (or diagnostic) concentration** | Concentration of an insecticide that, in a standard period of exposure, is used to discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population. It is expressed as a percentage of an active ingredient per unit volume of a carrier oil that is applied in a fixed amount per unit area on the test paper.<br>*Note: A discriminating concentration combines a fixed exposure time and the amount of insecticide on a test paper, the uptake of which depends on the time of actual tarsal contacts.* |
| **insecticide discriminating (or diagnostic) dose for resistance** | A fixed dose of an insecticide ingredient dissolved in a solvent that is topically applied on the mosquito body; used to discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population.<br>*Note: Where the genetic factor for resistance is either dominant or recessive, only one discriminating dose operates. Where the factor is semidominant, two such doses may operate: a lower dose that kills susceptible mosquitoes only, and an upper dose that kills both susceptible and heterozygous (but not homozygous) resistant mosquitoes.* |
| **insecticide mixture** | An insecticide product comprising either two or more co-formulated active ingredients (AIs) or a product prepared as a tank mixture using two or more AIs so that, when applied, the mosquito will come into contact with both or all of them simultaneously. For resistance management, a mixture usually includes AIs of different classes. |

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|---|---|
| **insecticide mosaic** | A method for mitigating resistance, whereby insecticides with different modes of action are applied in separate parts of a surface area under coverage (usually in a grid pattern), so that parts of the mosquito populations are likely to be exposed to one insecticide and others to another insecticide of a different class.<br><br>*Note: Ideally, this method is combined with insecticide rotation, whereby the treatments of the mosaic are switched between parts periodically.* |
| **insecticide resistance** | Ability of mosquitoes to survive exposure to a standard dose of insecticide; this ability may be the result of physiological or behavioural adaptation.<br><br>*Note: The emergence of insecticide resistance in a vector population is an evolutionary phenomenon caused either by behavioural avoidance (e.g. exophily instead of endophily) or by physiological factors whereby the insecticide is metabolised, not potentiated, or absorbed less in resistant mosquitoes than in susceptible mosquitoes.* |
| ***Kdr* (knockdown mutation)** | Knockdown resistance is caused by a series of genes involving a mutation in the sodium ion channel, the target site of pyrethroids and organochlorine compounds (e.g. dichlorodiphenyltrichloroethane, DDT), and conferring resistance to these insecticides. |
| **larvicide** | A chemical substance applied to aquatic habitats to kill mosquito larvae.<br><br>*Note: Larvicides are applied in the form of oils or monolayer films (to asphyxiate larvae and pupae), or as formulations such as tablets (for direct application), granules, emulsifiable concentrates, water-dispersible granules or wettable powders.* |
| **net, insecticide-treated** | Mosquito net that repels, disables or kills mosquitoes that come into contact with the insecticide on the netting material. The two categories of insecticide-treated net are:<br><br>- *conventionally treated net:* a mosquito net that has been treated by dipping it into a WHO-recommended insecticide. To ensure its continued insecticidal effect, the net should be re-treated periodically.<br><br>- *long-lasting insecticidal net:* a factory-treated mosquito net made of netting material with insecticide incorporated within or bound around the fibres. The net must retain its effective biological activity for at least 20 WHO standard washes under laboratory conditions and 3 years of recommended use under field conditions.<br><br>*Note: Untreated mosquito nets can also provide substantial protection against mosquito bites, but they have less effect against vectorial capacity and transmission rates..* |
| **susceptible population** | A population that has not been subjected to insecticidal pressure and in which resistant individuals are either absent or rare. |

| | |
|---|---|
| **susceptibility tests** | Bioassays in which samples of insects from a wild population are exposed to a fixed concentration of insecticide on test papers designed to reliably kill susceptible insects, so that any survivors may be assumed to be resistant. The WHO standard tube-test method is long established, whereas the bottle bioassay method has been developed more recently by the US Centers for Disease Control and Prevention. |
| **sympatry** | Occurs when species occupy roughly the same area of land at the same time but do not interbreed. |
| **synergist** | A substance that does not itself have insecticidal properties, but which, when mixed and applied with insecticides of a particular class, considerably enhances their potency by inhibiting an enzyme that normally acts to detoxify the insecticide in the insect system. |

# 1. INTRODUCTION

Global malaria control efforts have produced remarkable results over the past 15 years. In 2016, there were an estimated 216 million cases of malaria and an estimated 445 000 deaths worldwide. Between 2010 and 2016, malaria incidence was reduced by 18% globally and by 20% in Africa *(1)*. Much of the recent decrease in the global malaria burden has been achieved through the scale-up of core vector-control interventions, namely long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) with insecticides *(2)*.

Five classes of insecticide have so far been recommended by WHO for use against adult mosquitoes in public health programmes. Among these, pyrethroids have been the most extensively used, although recently, IRS programmes have significantly reduced their dependence on pyrethroids because of the emergence of resistance. The pyrethroids offer several advantages over other insecticide classes in terms of cost, human safety (low toxicity to mammals) and duration of residual action. They are widely used in agriculture and as household pesticide products; however, their use as larvicides is limited because of their high toxicity to nontarget aquatic organisms including fish. Currently, pyrethroids are used in all WHO prequalified LLINs and in several IRS products.[1] There is at the moment no alternative to the treatment of nets, although pyrethroid plus synergist nets are now available. Products currently under evaluation by WHO include LLINs with a pyrethroid and another compound from an unrelated class (e.g. chlorfenapyr or pyriproxyfen), and an IRS product containing chlorfenapyr.

The near ubiquitous use of pyrethroids for the treatment of nets and the long use of certain other classes – such as organochlorine compounds (e.g. dichlorodiphenyltrichloroethane, DDT) and organophosphate compounds – for IRS are likely to have contributed to the development of resistance against the pyrethroids in malaria vectors. Following an increase in entomological surveillance in malaria affected regions in recent years, significant amounts of data have now been collected by WHO to confirm already strong suspicions that insecticide resistance is now widespread in many malaria vectors throughout the world, and is of particular concern in African vectors especially *An. funestus (3)*. Since 2010, resistance to at least one class of insecticides has been reported in at least one malaria vector species in 60 of the 96 malaria-endemic countries that conducted monitoring; also, 49 countries reported resistance to at least two classes of insecticide. Resistance to all four available classes of insecticide has been reported. Resistance to pyrethroids was most commonly reported, with three quarters of countries that monitored this class in 2014 reporting resistance *(4)*. The mechanisms responsible for the now widespread frequency of resistance have also been identified. These tend to be of two main types: those mediated by changes at the target site of the insecticide (e.g. knockdown resistance [*kdr*] mutations) and those caused by increases in the rate of insecticide metabolism. However, it is likely that other, as yet unknown, resistance mechanisms are contributing to the strong resistance phenotypes seen in some populations. Resistance mechanisms and their implications for vector-control strategies are explained further in Box 1.1.

1 http://www.who.int/pq-vector-control/prequalified-lists/

The global malaria community is responding to the potential threat posed by emerging insecticide resistance. In May 2012, WHO launched the Global plan for insecticide resistance management in malaria vectors (GPIRM) *(5)*, which sets out a comprehensive framework for action in five key areas (or "pillars"):

- planning and implementing national insecticide resistance management strategies;
- ensuring entomological and susceptibility monitoring and effective data management;
- developing new, innovative vector-control tools;
- filling knowledge gaps on resistance mechanisms and the impact of current insecticide resistance management approaches; and
- enabling mechanisms to improve advocacy and build human and financial resources.

The GPIRM is unequivocal about the need for an intensification of the insecticide resistance monitoring effort. It also calls for greater regularization of this function within national malaria control programmes (NMCPs). In particular, monitoring plans increasingly need to address the requirement for more detailed data on vector species distributions and their relevant attributes (e.g. biting and resting behaviours), the resistance status of each vector species or population to insecticides currently used or planned for use, and the quality and efficacy of vector-control interventions. Epidemiological studies that assess the operational implications of different types of resistance are also seen as a vital part of the expanded knowledge base that is now urgently needed to guide insecticide use, and of the development of strategies for managing insecticide resistance as part of malaria and other vector-borne disease control programmes *(6)*.

WHO has been providing support to countries in monitoring and managing insecticide resistance, and this remains one of the core functions of its Global Malaria Programme (GMP). The organization has served as the global coordinator for information on vector resistance for more than 50 years, providing Member States with regularly updated advice and guidance on monitoring and managing insecticide resistance as it evolves. As part of this role, and to ensure comparability of insecticide resistance data from different countries and sources, WHO has developed standard test procedures and operational standards for detecting and monitoring insecticide resistance in a range of disease vectors, including mosquitoes. The supply of quality-assured susceptibility test kits for use in the field has also been a core component of WHO's work in this area.

In the case of the malaria vectors, a series of documents with instructions for testing for the presence of insecticide resistance using a standardized bioassay technique in adult mosquitoes have been published *(7-10)*. Successive updates of the procedures have reflected developments in malaria control strategies; in particular, the introduction of new classes of insecticides and new insecticides within existing classes in vector-borne disease control programmes. The latest version of these procedures, published in 2013 *(9)*, covers four of the main classes of insecticides in common use. These insecticides are organochlorine (e.g. DDT), organophosphate (e.g. malathion and pirimiphos-methyl), carbamate (e.g. bendiocarb) and pyrethroid (e.g. deltamethrin, permethrin) insecticides, with new compounds representing the pyrroles (e.g. chlorfenapyr) and phenyl pyrazoles (e.g. fipronil).

Given the mounting evidence of increasing resistance among malaria vectors, especially to pyrethroids, and the desire not to undermine recent gains made in the battle against malaria, attention has focused on the need for more intensive and improved monitoring of insecticide resistance (especially the operational and epidemiological significance of resistance where it occurs). The aim is to guide development of national insecticide resistance management strategies. This has prompted calls for an update of the 2013 guidelines, primarily to make them more operationally useful in terms of vector-control decision-making and policies.

BOX 1.1

### INSECTICIDE RESISTANCE MECHANISMS: IMPLICATIONS FOR VECTOR-CONTROL PROGRAMMES

The management of insecticide resistance is complicated by the fact that resistance takes a variety of forms. Broadly speaking, the primary resistance mechanisms can be divided into two groups: *metabolic resistance* and *target-site resistance*.

Metabolic resistance arises because of changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than normal. The detoxification prevents the insecticide from reaching the intended site of action within the mosquito. In the case of malaria vectors, three enzyme systems are believed to be important metabolizers of insecticides: esterases, monooxygenases and glutathione *S*-transferases.

Target-site resistance occurs when the protein receptor that the insecticide is designed to attack is altered by a mutation. When this happens, the insecticide can no longer bind to the intended target site of the receptor; thus, the insect is either unaffected or is less affected by the insecticide. In the case of DDT and the pyrethroids, the mutation occurs in the sodium channel receptor, conferring what is described as "knockdown resistance" (mediated by the *kdr* genes). In the case of the organophosphates and the carbamates, the mutation occurs in the protein acetylcholinesterase (a neurotransmitter), conferring what is usually referred to as *Ace-1* resistance. The gene for resistance to dieldrin (*rdl*) occurs in the gamma aminobutyric acid receptor and has been shown to also confer resistance to fipronil.

An added complication arises because of "cross-resistance" between different classes of insecticides that share the same mode of action. Thus, vectors that are resistant to DDT because they possess the *kdr* resistance-associated gene will probably also be resistant to certain pyrethroid insecticides. Likewise, the *Ace-1* mutation can confer target-site resistance to both carbamate and organophosphate insecticides. Cross-resistance can also occur when insecticides of two or more classes of insecticides are metabolized by the same enzyme. Furthermore, the prevalence of multiple insecticide resistance mechanisms that co-occur in single populations and even in individual mosquitoes is increasing in malaria affected countries. The existence of cross-resistance and multiple resistance restricts the choice of alternative insecticides in situations where resistance has been detected.

What impact the observed spread of resistance will have on the effectiveness of current vector-control programmes is, however, far from certain. A 2014 review found that, even in the presence of pyrethroid resistance, insecticide-treated mosquito nets (ITNs) perform better than untreated nets in terms of protection against blood-feeding, and ITNs can induce significant mosquito mortality *(11)*. However, since then, the frequency of pyrethroid resistance has increased in many settings *(3)*. In general, the limited data available indicate that high frequencies or intensities of resistance can lead to failure of IRS and can thereby have an epidemiologically significant effect on malaria incidence *(12)*. For the efficacy of LLINs, the situation is more complex, and conclusive evidence of control failure due to pyrethroid resistance is still lacking, with a large multi-country evaluation finding no evidence of an association between malaria disease burden and pyrethroid resistance in areas where LLINs were used *(13)*. Nevertheless, the possibility that the increasing intensity of resistance being selected in field populations of mosquitoes will reduce the efficacy of pyrethroid-based interventions cannot be ignored. The prudent course of action is therefore to adopt a proactive approach and modify current practices so as to delay the spread of resistance and preserve the effectiveness of current insecticides, at least until novel tools based on new classes of insecticides are available. It is envisaged that, through use of resistance intensity assays, it will be possible to identify regions and areas where resistance is most intensively expressed and where insecticide-based vector control is most likely to fail, and therefore where an urgent response is required.

## 2. EVOLUTION OF THE WHO INSECTICIDE SUSCEPTIBILITY TEST: BACKGROUND TO CURRENT REVISION

The WHO insecticide susceptibility bioassay that is the main subject of these revised and expanded procedures is a simple direct response-to-exposure test. Mosquitoes are exposed to known concentrations of an insecticide for a fixed period of time, and the number of fatalities is recorded at least 24 hours after exposure. In its present form, the test is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes. Thus, the test is intended to be used as a field and laboratory surveillance tool, with the limitation that it gives little information on the underlying mechanism(s) conferring resistance (where detected) or the strength of that resistance.

The test equipment and method has changed relatively little since WHO first recommended the use of a standard bioassay technique to detect insecticide resistance in the early 1960s *(14)*. Any methodological changes that have been made over the years have been fairly minor, relating largely to the test conditions and controls, and to the insect sampling protocols *(7, 10, 15)*. Changes incorporated into the 2013 update of the guidelines *(9)* related primarily to data interpretation criteria, the addition of discriminating concentrations for certain new insecticide active ingredients, a brief description of tests for resistance mechanisms and a synopsis of the bottle bioassay developed by the US Centers for Disease Control and Prevention (CDC).[2]

The original impetus to develop the 2013 version of the guidelines *(9)* arose from recommendations of an informal WHO-GMP consultation convened in May 2010. The consultation reviewed the current status of insecticide resistance in malaria vectors in order to identify strategies for delaying the emergence of resistance. The impetus for the current revision stems from an informal consultation convened in December 2015 to revise and adapt the current procedures. The aim is to provide a stronger focus on producing operationally meaningful data to enable policy-making decisions for vector control. Recognizing that insecticide resistance has a central role in monitoring the effective planning and implementation of insecticide-based vector control, and taking into account resistance management, a WHO consultation in April 2016 made several recommendations about the future direction of insecticide resistance detection and monitoring. In addition to highlighting the need for an immediate scale-up in susceptibility testing and the establishment of data reporting mechanisms, the consultation recommended an expanded three-step bioassay protocol:

- Step 1: Detect the presence of insecticide resistance phenotypes in a population using discriminating concentration bioassays, according to the method outlined in the 2013 publication *(9)*.
- Step 2: Assess the strength of phenotypic resistance by performing bioassays using five and 10 times (5× and 10×) the discriminating concentrations of insecticides.
- Step 3: Determine the involvement of metabolic resistance mechanisms by assessing the effect of a synergist such as piperonyl butoxide (PBO) on the resistance phenotypes detected in Steps 1 and 2.

2 The bottle bioassay is a complementary method for field testing of insecticide resistance using diagnostic and intensity concentrations *(16)*.

This expanded version is intended to make it easier to generate comprehensive data that will be useful in making decisions on how to respond to the presence of insecticide resistance. The CDC bottle bioassay offers options to test 1×, 2×, 5× and 10× concentrations. Data from these bioassays increase confidence that multiples of the discriminating concentrations are informative and are applicable to WHO susceptibility bioassays.

WHO continues to recommend that the standard WHO susceptibility tests should continue to be a primary method by which resistance is detected. However, it was considered necessary to update the existing resistance-monitoring procedures *(9)* to also highlight the need for operationally meaningful data. Consequently, a technical consultation was convened to inform updates to the procedures. Specific objectives identified were to:

- expand current WHO test procedures to generate more operationally meaningful data for monitoring insecticide resistance in malaria vectors in order to align with new developments in insecticide resistance management;
- provide procedures for resistance intensity bioassays using 5× and 10× the discriminating concentrations;
- advise on test procedures for assessing the effect of synergists (e.g. PBO) on the expression of resistance phenotypes; and
- provide an updated list of "discriminating concentrations" for adult mosquitoes for the insecticides recommended for use in malaria vector control.

Insecticide resistance is a quantitative trait that varies in its intensity. Therefore, it is further recommended that routine susceptibility monitoring using the expanded WHO insecticide susceptibility test be supplemented by additional genetic testing (polymerase chain reaction, PCR) and biochemical testing (enzyme activity assays). Supplementary test methods for determining the underlying mechanisms of resistance, their intensities of expression and tools for tracking the spread of resistance are important for decision-making to manage insecticide resistance.

The procedures for larvicide susceptibility testing are still considered valid; hence, they are not repeated in the present document. Those interested in the evaluation of larvicides are advised to refer to the original documents, which are available from WHO *(8, 17)*. However, the CDC bottle bioassay procedure is discussed in Section 5.

# 3. WHO SUSCEPTIBILITY TEST FOR ADULT MOSQUITOES

The WHO susceptibility bioassay is a direct response-to-exposure test. It measures mosquito mortality to a known standard concentration of a given insecticide, either with a discriminating concentration or with intensity concentrations. The test procedure itself is summarized in Box 3.1.

## 3.1 Susceptibility tests with discriminating concentration

The concept of discriminating (or diagnostic) concentration is now well established, and has been widely adopted for the purposes of testing and monitoring insecticide resistance in mosquitoes and other disease vectors *(9, 10, 18, 19)*. The use of discriminating concentrations in routine insecticide resistance monitoring is explained in Box 3.2.

Discriminating concentrations have been established under standardized laboratory conditions for all insecticides currently in use in malaria control programmes. They have been reported in previous versions of the guidelines *(7, 9, 10, 15)* and are updated again in this revision. Discriminating concentrations for a range of pyrethroid insecticides were included for the first time in the guidelines published in 1998 following a multicentre study *(15)*. The anopheline species used in that study were *An. aconitus*, *An. albimanus*, *An. arabiensis*, *An. dirus*, *An. freeborni*, *An. gambiae s.s.*, *An. maculatus*, *An. minimus* and *An. stephensi*. Since then, discriminating concentrations have been established for a further four insecticide compounds, although as yet these are tentative, pending confirmation by WHOPES through a multicentre validation.

Insecticide susceptibility test papers impregnated with insecticides at the appropriate discriminating concentration are supplied as part of the WHO test kits manufactured by Universiti Sains Malaysia and coordinated by WHO (see also Section 3.7.3). To be certain that all susceptible mosquitoes are killed, WHO has applied the use of discriminating concentrations in one of the following two ways:

- twice the lowest concentration that gave systematically 100% mortality (i.e. $LC_{100}$) after 60 minutes exposure and a holding period of 24 hours of a susceptible laboratory strain or a susceptible field population of mosquitoes; or
- twice the $LC_{99.9}$ values determined by baseline susceptibility testing against a susceptible laboratory strain or a susceptible field population of mosquitoes.

A susceptible population is one that has not been subjected to insecticidal pressure and in which resistant individuals are either absent or rare.

Table 3.1 lists the WHO recommended discriminating and intensity bioassay concentrations of insecticides for determining susceptibility of adult anopheline mosquitoes using WHO insecticide susceptibility tests. It also gives the concentrations for synergists for synergist-insecticide bioassays. Box 3.1 provides the methodology for carrying out the bioassays.

In most countries, insecticide resistance monitoring for other vectors (e.g. *Aedes* mosquitoes) is also necessary. Given that the methodology is the same as for *Anopheles* mosquitoes, a table with the discriminating concentrations and exposure time of insecticides commonly used for *Aedes* mosquitoes is provided in Annex 4.

## 3.2 Susceptibility tests for determining intensity of resistance

It was decided to incorporate certain insecticides at 5× and 10× the discriminating concentrations into the WHO susceptibility test for adult mosquitoes because resistance phenotypes detected using the discriminating concentrations do not necessarily provide information in terms of efficacy failure of that insecticide in the field. It was also suggested that any resistance phenotypes detected using the discriminating concentrations should be further assessed for their potential operational significance by exposing subsequent mosquito samples from the same target vector population to substantially higher concentrations of the pertinent insecticides. Although these higher concentrations for each insecticide will not correspond to their recommended field application rates, they will yield relevant information about the intensity of resistance, or the "strength" of expression of the resistance phenotype(s) in question. This information can then be used to inform operational decisions such as a change of insecticide for IRS or the introduction of a nonpyrethroid for IRS in areas with LLINs as the main intervention.

The WHO susceptibility test for adult mosquitoes has thus been expanded to also include the use of 5× and 10× the discriminating concentrations in a stepwise manner. The aim is to provide information on the range (if any) of resistance phenotypes present in a target vector population and their potential operational significance. A flowchart illustrating criteria for each successive step is shown in Fig. 3.1. Table 3.1 includes 5× and 10× concentrations for only certain insecticide compounds that are either in most demand or for which preparation of test papers seems technically feasible.

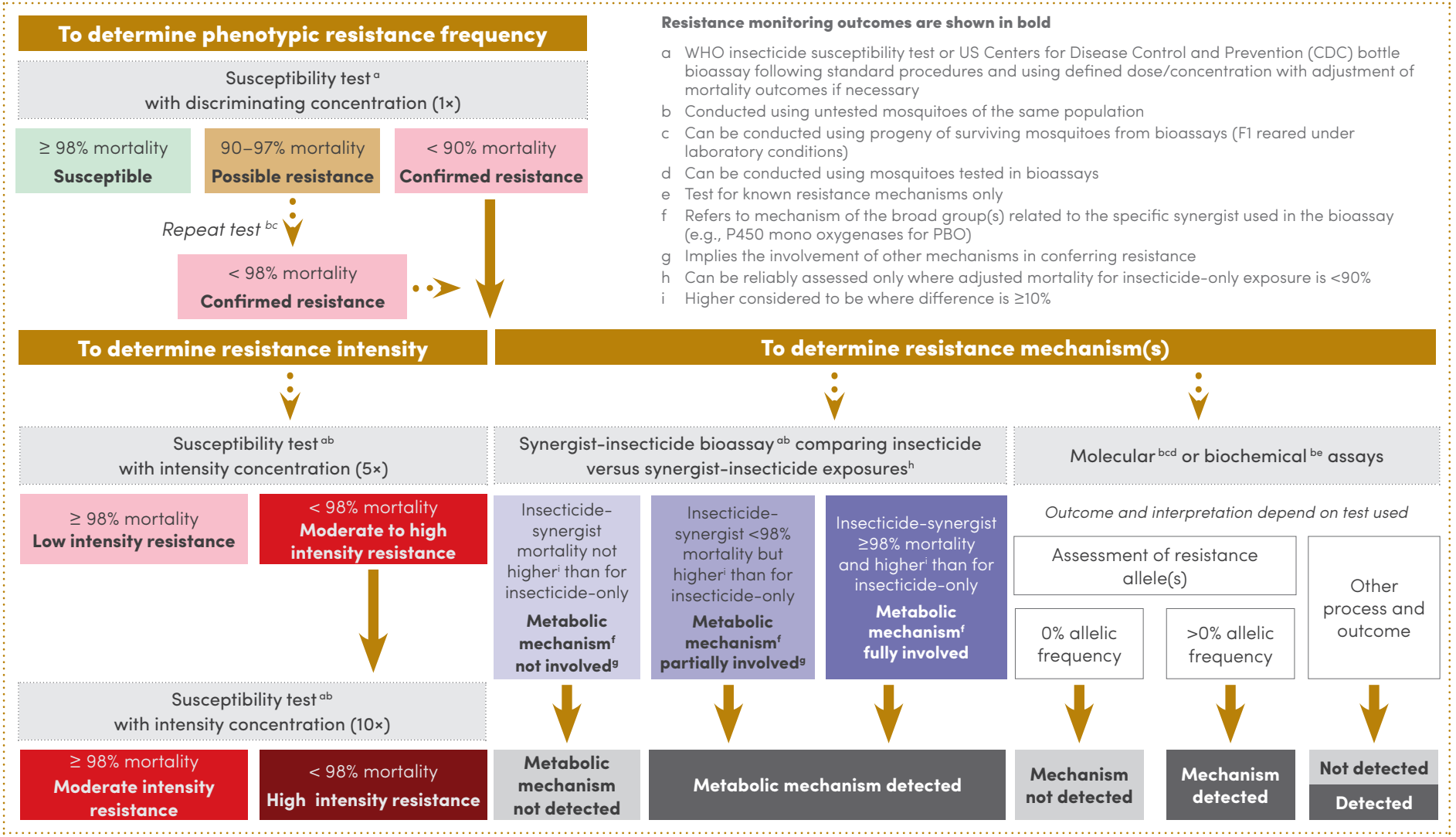


FIG. 3.1

**Overview of process and outcomes for insecticide resistance monitoring in malaria vector mosquitoes. Includes measures of: a) phenotypic resistance frequency via discriminating concentration bioassays, b) resistance intensity via intensity concentration bioassays, and c) resistance mechanisms via synergist-insecticide bioassays, molecular and biochemical assays**

TABLE 3.1

**Discriminating and intensity bioassay concentrations of insecticides for determining susceptibility of adult anopheline mosquitoes using WHO insecticide susceptibility tests, and concentration of piperonyl butoxide as a synergist for synergist-insecticide bioassays**

| INSECTICIDE CLASS | INSECTICIDE | DISCRIMINATING CONCENTRATION (%) (1-HOUR EXPOSURE PERIOD) | 5×[a] CONCENTRATION (%) (1-HOUR EXPOSURE) | 10×[a] CONCENTRATION (%) (1-HOUR EXPOSURE) | CONTROL PAPER | REMARKS |
|---|---|---|---|---|---|---|
| Carbamates | Bendiocarb | 0.1 | 0.5 | 1 | Olive oil | |
| | Carbosulfan | 0.4 | | | Olive oil | Tentative, to be confirmed by WHOPES. Based on data published by N'Guessan et al. (2003) *(20)* and Ahoua Alou et al. (2010) *(21)*. |
| | Propoxur | 0.1 | | | Olive oil | Insecticides that need to be tested for resistance whenever possible; does not necessarily mean that the insecticides are recommended by WHO for use in malaria vector control. |
| Organochlorines | DDT | 4 | | | Risella oil | |
| | Dieldrin | 0.4 | | | Risella oil | Resistance to dieldrin may be tested for whenever possible; does not necessarily mean that dieldrin is recommended by WHO for use in malaria vector control. Exposure to dieldrin at 0.4% kills susceptible (SS) individuals but not resistant heterozygotes (RS); exposures to dieldrin at 4% kills heterozygotes (RS) but not homozygous (RR) resistant individuals. |
| | | 4 | | | Risella oil | |
| Organophosphates | Fenitrothion | 1 | | | Olive oil | Two-hour exposure time. |
| | Malathion | 5 | | | Olive oil | |
| | Pirimiphos-methyl | 0.25 | 1.25 | 2.5 | Olive oil | Tentative and based on unpublished industry data, 2006; to be confirmed by WHOPES. |
| Pyrethroids | Alpha-cypermethrin | 0.05 | 0.25 | 0.5 | Silicone oil | Tentative; to be confirmed by WHOPES. |
| | Cyfluthrin | 0.15 | 0.75 | 1.5 | Silicone oil | |
| | Deltamethrin | 0.05 | 0.25 | 0.5 | Silicone oil | |

| INSECTICIDE CLASS | INSECTICIDE | DISCRIMINATING CONCENTRATION (%) (1-HOUR EXPOSURE PERIOD) | 5×[a] CONCENTRATION (%) (1-HOUR EXPOSURE) | 10×[a] CONCENTRATION (%) (1-HOUR EXPOSURE) | CONTROL PAPER | REMARKS |
|---|---|---|---|---|---|---|
| Pyrethroids | Etofenprox | 0.5 | 2.5 | 5 | Silicone oil | |
| | Lambda-cyhalothrin | 0.05 | 0.25 | 0.5 | Silicone oil | |
| | Permethrin | 0.75 | 3.75 | 7.5 | Silicone oil | |
| Synergist | Piperonyl butoxide | 4 | | | Silicone oil | |

DDT, dichlorodiphenyltrichloroethane; WHOPES, World Health Organization Pesticide Evaluation Scheme

[a] Stability and use of higher concentrations is currently not validated by WHOPES. Higher concentrations are only proposed for some widely used insecticides. It may not be possible to treat filter papers at some of the higher concentrations owing to solubility limits.

Note: For the insecticides clothianidin (neonicotinoid) and chlorphenapyr (pyrrole), no discriminating concentrations are available yet.

Sources: Based on WHO 1992 *(19)* and WHO 1998 *(10)* unless otherwise specified.

BOX 3.1

## MEASURING SUSCEPTIBILITY TO INSECTICIDES IN ADULT MOSQUITOES: THE WHO INSECTICIDE SUSCEPTIBILITY TEST PROCEDURES FOR DISCRIMINATING, 5× AND 10× CONCENTRATIONS

1. The investigator puts on gloves. Six sheets of clean white paper (12 × 15 cm), rolled into a cylinder shape, are inserted into six holding tubes (with the green dot), one per tube, and fastened into position against the wall of the tube with a steel spring wire clip. The slide unit is attached to the tubes at the other end.

2. Ideally, 120–150 active female mosquitoes are aspirated (in batches) from a mosquito cage into the six green-dotted holding tubes through the filling hole in the slide, to give six replicate samples of 20–25 mosquitoes per tube.

3. Once the mosquitoes have been transferred, the slide unit is closed and the holding tubes set in an upright position for 1 hour. At the end of this time, any moribund mosquitoes (i.e. those unable to fly) and dead mosquitoes are removed.[a]

4. The investigator inserts one oil-treated paper (the control) into each of two yellow-dotted tubes, ensuring that the label of the paper is visible on the outside of the tube. The paper is fastened with a copper clip and the tube closed with a screw cap.

5. Four exposure tubes with red dots are prepared in much the same way as the yellow-dotted tubes. Each of the four red-dotted exposure tubes is lined with a sheet of insecticide-impregnated paper such that print label is visible on the outside. Each paper is then fastened into its position against the wall with a copper spring-wire clip and the tube is closed with a screw cap.

6. The empty exposure tubes are attached to the vacant position on the slides and, with the slide unit open, the mosquitoes are blown gently into the exposure tubes. Once all the mosquitoes are in the exposure tubes, the slide unit is closed (usually a cotton wool plug is inserted into the hole to lock the slide) and the holding tubes are detached and set aside. The investigator now removes the gloves.

7. Mosquitoes are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (unless otherwise specified). The tubes are placed in an area of reduced lighting or covered with cardboard discs to reduce light intensity and to discourage test mosquitoes from resting on the mesh-screen lid.

8. At the end of the 1-hour exposure period (or longer for certain compounds, as outlined in Table 3.1), the mosquitoes are transferred back to the holding tubes by reversing the procedure outlined in Step 6. The exposure tubes are detached from the slide units. A pad of a cotton wool soaked in 10% sugar water is placed on the mesh-screen end of the holding tubes.

9. Mosquitoes are maintained in the holding tubes for 24 hours (or longer for slow-acting compounds). During this time, it is important to keep the holding tubes in a shady, sheltered place in the laboratory or in a chamber maintained at 27 °C ± 2 °C temperature and 75% ± 10% relative humidity. Temperature and humidity should be recorded during the recovery period.

10. At the end of recovery period (i.e. 24 hours post-exposure or longer for slow-acting compounds), the number of dead mosquitoes[a] is counted and recorded. An adult mosquito is considered to be alive if it is able to fly, regardless of the number of legs remaining. Any knocked down mosquitoes, whether or not they have lost legs or wings, are considered moribund and are counted as dead. A mosquito is classified as dead or knocked down if it is immobile or unable to stand or take off.

On completion of the susceptibility test, mosquitoes may be transferred to individual, clearly labelled microcentrifuge tubes with a lid for airtight locking (separating dead and live mosquitoes into separate tubes) for preservation until such time as they can be transferred to suitable facilities for species identification and supplementary testing if necessary. A schematic representation of the procedure is shown in Fig. 3.2.

[a] For the purpose of insecticide bioassays, the definition of knockdown and mortality involves not only the state of the insect but also the time at which the observation is made. A mosquito is classified as "dead" or "knocked down" if it is immobile or unable to stand or take off. The distinction between knocked down and dead is defined only by the time of observation. The assessment of knockdown is made within 1 hour of exposure. Mortality is determined at least 24 hours after exposure. The holding container may be tapped a few times before a final determination is made. In the case of slow-acting insecticides, the recovery (holding) period may be extended beyond 24 hours. Control mortality should be measured over the same recovery period. Mortality after 24 hours should be recorded; in some cases, repeated observations may be appropriate. Classification of adult mosquitoes as alive, knocked down or dead in bioassays is summarized below:

| ALIVE | KNOCKED DOWN OR DEAD AFTER EXPOSURE | |
|---|---|---|
| | MORIBUND | DEAD |
| Can both stand and fly in a coordinated manner | • Cannot stand (e.g. has only one or two legs)<br>• Cannot fly in a coordinated manner<br>• Lies on its back, moving legs and wings but unable to take off<br>• Can stand and take off briefly but rapidly falls down | • No sign of life<br>• Immobile<br>• Cannot stand |

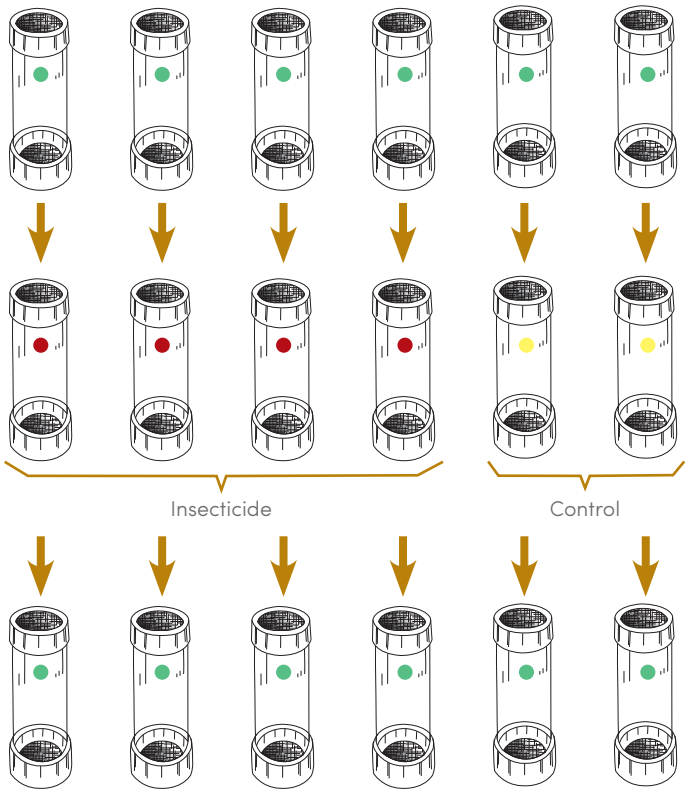


FIG. 3.2
**Steps to perform the WHO insecticide susceptibility test for discriminating, 5× and 10× concentrations**

BOX 3.2
## DETERMINING RESISTANCE IN VECTOR POPULATIONS

### Use of discriminating concentrations

To determine resistance in vector populations it is necessary to first establish baseline susceptibility data for individual insecticides in a normal or "susceptible" population of a given species. This is achieved by exposing nonresistant vectors to filter papers impregnated with serial concentrations of a given insecticide compound, and plotting the percentage mortality against exposure on logarithmic-probability paper. The graph can be used to estimate the concentrations required to produce various levels of kill; this calculation can also be done using a log-probit statistical model. Using this method, it is possible to derive the concentration corresponding to 99.9% mortality (the $LC_{99.9}$ value); at this concentration there is a high probability that all individuals in a susceptible population will be killed. Double this concentration is conventionally known as the discriminating (or diagnostic) concentration (i.e. 1×). Once discriminating concentrations for individual insecticides have been established under standardized laboratory conditions

using known susceptible strains or populations of a range of mosquito vector species, it is not necessary for routine monitoring purposes to conduct susceptibility tests at the full range of test concentrations. Instead, it is sufficient to conduct a standard bioassay resistance test using the discriminating concentration, because any survivors at this concentration may be considered to be resistant. This approach has obvious advantages in terms of the feasibility, cost and efficiency of testing. However, confirmation of resistance using discriminating concentrations may not necessarily correlate with operational failure of insecticide formulations used for IRS, or for ITNs or LLINs. Further assays designed to assess the extent of resistance intensity can be used to inform operational decisions (see below). Discriminating concentrations for various insecticide compounds either used in vector control or evaluated through research have been established for different mosquito species (Table 3.1). For new insecticide compounds, in cases where mosquito species are not routinely monitored or in specific situations where baseline data are not available, it is necessary to first establish the baseline susceptibility as described above.

**Use of 5× and 10× intensity concentrations**

Any resistance phenotypes detected using the discriminating insecticide concentrations can be assessed for their potential operational significance by exposing subsequent or additional mosquito samples to the applicable 5× and 10× higher concentrations of those insecticides. Exposures at the higher concentrations will yield information on the intensity of resistance, which can be defined as the "strength" of a resistance phenotype. It is suggested that resistance at 5× and especially at 10× the discriminating concentration may indicate or predict operational control failure and highlight a particularly urgent need to develop an appropriate resistance management strategy *(5)*. Instructions on how to interpret these results can be found in Section 3.6.

## 3.3 Sampling protocols

### 3.3.1 Selection of test specimens

The age, physiological status and gender of mosquitoes are important factors that can influence the results of insecticide susceptibility tests. The use of males is not recommended for resistance monitoring because they are usually smaller, have a shorter life expectancy and are more fragile than females, and therefore tend to have higher control mortalities. Susceptibility testing is thus conducted using only female mosquitoes.

Studies using adult female mosquitoes have repeatedly shown that both age and physiological status (i.e. unfed or blood fed, semi-gravid or gravid) have a marked effect on susceptibility to insecticides. For instance, older mosquitoes are sometimes less resistant to insecticides, especially when resistance is conferred by the presence of a detoxifying enzyme, the activity of which may decline with age *(24)*. Consequently, it is recommended that initial susceptibility tests using discriminating concentrations be performed on adult females aged 3–5 days that are nonblood fed (i.e. sugar fed and starved for about 6 hours). Assays using the 5× and 10× concentrations can be performed using nonblood-fed females. Under field conditions it is easier to conduct tests using older females that are wild-caught, because older blood-seeking females are the epidemiologically important cohort of vector populations.

In the interests of obtaining age-standardized results, it is recommended that susceptibility tests be performed using either adult females derived from larval collections (the preferred option) or, if larval collections are not possible, the F1 progeny of wild-caught female mosquitoes. If using larval collections, samples from the same place and the same type of breeding site may be pooled before testing in order to provide a sufficient number of test insects. However, larval collections should ideally be made from a number of different breeding sites; this avoids sampling individuals from single egg batches, which could result in a high proportion of siblings in the test population. Similarly, the genotypic variability of the progeny of one adult female is limited, so wild-caught females should ideally be collected from a number of different locations in order to ensure a broadly representative sample of the local population. In practice, this means that at least 30 batches of eggs (or more if there is a mixture of species) should be reared from the wild-caught females.

When relying on larval collections to provide young adult females for resistance monitoring, it is important to record the type of breeding site (e.g. rice field, rainwater collection, irrigation channel or well) and the global positioning system (GPS) coordinates from which the larval collection was made because:

- exposure to pesticide residues will vary with type of breeding site; and
- some taxa within the same species complex will preferentially discriminate between breeding sites (e.g. *An. coluzzii* is more likely to breed in rice fields whereas *An. gambiae s.s.* tends to predominate in rainwater collections).

A third option is to use wild-caught females directly. In this case, it is necessary to select and test only unfed females. If necessary, females may be sustained with sugar water and then starved for a few hours before the tests.

The main advantage of using wild-caught females directly is convenience and the fact that they are the operationally relevant population. The main disadvantage is that their age is unknown, which may lead to greater variation in susceptibility test results (and probably an underestimation of resistance) depending on the species distribution and the insecticide being tested. The relative merits of using F1 progeny of wild-caught females and wild-caught females directly are compared in Table 3.2.

TABLE 3.2
**Advantages and disadvantages of using F1 progeny and wild-caught females for bioassays**

| SAMPLE | ADVANTAGES | DISADVANTAGES |
|---|---|---|
| F1 progeny | Age of vectors can be kept constant between tests, allowing results from different times and places to be compared.<br><br>Can be used in areas with low mosquito density even if it is not possible to catch sufficient numbers of wild adult female mosquitoes. | Requires better entomological facilities, which limits where the tests can be carried out.<br><br>Insectary conditions will differ from those in the natural environment.<br><br>Since many eggs may be derived from just a few adult females, the number of genomes sampled from the wild population is likely to be less than the number of insects tested. |
| Wild-caught females | Fewer facilities are required, so assays can be carried out in a greater number of locations.<br><br>Changes in susceptibility will more closely reflect the changes in intervention efficacy seen in the field.<br><br>The age distribution of the vectors should be representative of the wild vector population at a given time and location. | Age distribution and physiological condition of vectors will vary between samples, reducing the comparability of results.<br><br>Mosquitoes may have had prior nonlethal exposure to insecticide.<br><br>Wild-caught females are potentially infective and should be handled with care. |

Source: Adapted from Mnzava et al. (2015) *(4)*.

### 3.3.2 Spatial distribution and frequency of susceptibility testing

Previous editions of these guidelines did not make specific recommendations about the timing and frequency of susceptibility testing, but did note that comparisons of test data from a sentinel site over time are useful for assessing temporal trends in resistance. Comparisons of test data from multiple sites provide helpful information about the geographical distribution of resistance.

The resistance frequencies, the prevalence of resistance mechanisms and the population distribution of different taxa in a single locality vary markedly with time. Hence, the current recommendation remains; that is, that vector susceptibility to relevant classes of insecticides recommended for use by WHO be tested at least once per year, or in accordance with the changing seasons (which can affect vector species composition, relative abundances and susceptibility profiles) or the calendar of agricultural crops. Vector surveillance personnel should prioritize insecticide classes to be assessed based on those that are in use locally or are being considered for use, depending on the availability of mosquito samples. Considering the timing and frequency of susceptibility testing, the following are proposed as possible strategies:

- To determine the distribution of resistance, insecticide resistance monitoring should be conducted across a network of sentinel sites that have been selected to represent the range of vector species, eco-epidemiological zones and malaria transmission intensities that occur within a given country. In pre-elimination and elimination settings, surveillance should be prioritized in

transmission foci and areas of localized outbreaks, or following upsurges in disease cases.

- Testing could be repeated at the same sites in order to monitor changes in mosquito susceptibility over time, depending on the size or density of the vector population.
- Areas where the same insecticide is used for both vector control and agricultural purposes may require a more intensive monitoring schedule because of the potential for additional selection pressure on vector populations from agricultural use.
- Considering the heterogeneity in insecticide resistance distribution, an alternative to a sentinel site network is to conduct regular monitoring at district or county levels, or to monitor resistance along appropriately identified transects.

### 3.3.3 Sample size

Ideally, 120–150 adult female mosquitoes of a given species are required to conduct a single set of WHO insecticide susceptibility tests; of these, 100 will be exposed to the insecticide that is being tested (in four or five replicates each of around 20–25 mosquitoes). The remaining 50 mosquitoes will serve as "controls" (i.e. two replicates each of around 20–25 mosquitoes). If more than one insecticide is being tested, additional batches of mosquitoes will be required for each additional insecticide.

The control mosquitoes are exposed to papers impregnated with the appropriate carrier oil only; that is, without insecticide (see Table 3.1). In all other respects, the control mosquitoes are treated in the same way as the exposed mosquitoes (i.e. tested in parallel and under the same conditions). The controls are included to provide an estimate of natural mortality during the test and to account for all variables that may induce mortality other than the insecticide being tested. In this revision, testing using at least two controls (i.e. 40–50 mosquitoes) is recommended in order to improve the statistical significance of the results.

If it is not possible to collect enough mosquitoes on a single occasion (e.g. if working with wild-caught females) then it is possible to keep live mosquitoes until sufficient numbers have been collected. When using adults reared from larvae or F1 progeny, they can also be accumulated in cages until sufficient numbers have been obtained, keeping in mind the 3–5 day age range. When relying on pooled samples, mosquitoes should be provided with access to a sugar meal until a few hours before the bioassay is carried out.

In the event that insecticide resistance is suspected (i.e. there are survivors at the discriminating concentrations), it will be necessary to conduct further tests in order to identify the underlying resistance mechanism(s). This can be achieved using a combination of synergist bioassays and molecular or biochemical methods, or both (see Section 6). Fresh mosquito specimens are required for the biochemical tests; hence, it may be necessary to collect additional specimens or, if using larval collections, to reserve a subsample of the emergent adults (see Section 6).

### 3.3.4 Species identification

In many malaria-endemic regions, several species of mosquito belonging to the same group or complex tend to occur in sympatry. For instance, the *An. gambiae* species complex comprises eight cryptic species – *An. gambiae s.s.*, *An. coluzzii*, *An. arabiensis*, *An. bwambae*, *An. melas*, *An. merus*, *An. quadriannulatus* and *An. amharicus* – that are commonly found in sympatry with vector species, but are not all implicated in malaria transmission. Other examples of species complexes and groups include *An. culicifacies*, *An. fluviatilis*, *An. funestus*, *An. dirus*, *An. minimus*, *An. nuneztovari* and *An. albitarsis*. Different members of the same species complex do not necessarily share the same resistance mechanisms or exhibit the same insecticide resistance patterns. It is therefore essential that samples collected from the field be identified to the species level.

Morphological identification of species and species complexes or groups must be carried out after conducting the insecticide susceptibility tests. This allows the per cent mortality by species or species group to be properly calculated and, if necessary, the insects can be stored and appropriately labelled for later laboratory processing. The development of molecular techniques has made it possible to distinguish individual members of mosquito species complexes relatively quickly and easily using simple PCR-based assays. With this technique, species identification can be carried out after the bioassay, using specimens both dead and alive after exposure as well as those from the control group. Before identification by PCR, specimens should be stored on silica gel or in ethanol. To avoid DNA cross-contamination, individual mosquitoes can be placed in 0.5 ml microcentrifuge tubes.

Keys for morphological identification of collected mosquitoes are available for various regions. Appropriate molecular methods for identifying many malaria vectors to species level are given in the publication *Methods in Anopheles research manual (25)*.

It is best to identify all test mosquitoes to species level so that mortality can be calculated separately for each species. If it is not possible to identify all test mosquitoes, then a subsample of 40–50 mosquitoes per test can be used. The subsample should include all survivors plus some dead mosquitoes or, if there are many survivors, the subsample should be of survivors only. If F1 mosquitoes were used in tests, then it is sufficient to identify to species only the mothers of each family.

## 3.4 Test conditions and protocols

The steps involved in conducting the WHO susceptibility test have been described in Box 3.1. As mentioned, the basic procedure has remained more or less unchanged since it was recommended for use as a standard test for susceptibility in 1976 *(18)*. However, some minor modifications to the test protocols have been made over time. For example, the 1998 revision – when the pyrethroids were included for the first time – stipulated the need to maintain the holding tubes in a vertical position during the exposure and holding periods *(10)*.

In the 2013 revision *(9)* and in this current one, certain small changes to the recommended test conditions and protocols have been proposed, as described below. A standard data form for recording information about the susceptibility test, including details of the study area, the test specimens (the collection method, age, physiological status and species), the insecticide(s) under test and the test conditions, is provided at Annex 1.

### 3.4.1 Number of test mosquitoes

As mentioned above, 120–150 female mosquitoes should be tested for any insecticide at the discriminating concentration, with at least four replicates of 20–25 mosquitoes per test. When it is not possible to test this number of mosquitoes on a single day, tests can be conducted over a few days until this number is reached, provided that control tests are run in parallel. In this event, and to avoid multiple manipulations, impregnated papers can remain in the tubes, provided that they are wrapped in aluminium foil and kept at 4 °C between successive tests. A minimum of two controls (40–50 mosquitoes) is specified in this revision in order to improve the statistical validity of the results.

### 3.4.2 Ambient conditions

Ambient temperature can influence the toxicity of insecticides *(26)*, and relative humidity can affect the survival of mosquitoes during the holding period. Therefore, temperature and humidity need to be controlled during the test and holding periods. Ideally, tests should be carried out at 25 °C ± 2 °C and 80% ± 10% relative humidity. During the exposure and holding periods, both the temperature and relative humidity should be monitored, and the maximum and minimum values recorded at the start of the exposure period and again at the end of the holding period.

Throughout the test (exposure and holding periods), the exposure and holding tubes should be held in a vertical position for all insecticide classes (even for those insecticides that have a rapid knockdown effect, such as the pyrethroids). The mesh-screen ends of the exposure tubes should be covered with a piece of cardboard for the duration of the exposure to reduce light intensity. No tests should be conducted above a temperature of 30 °C. In the absence of an insectary or "field insectary" cool box, the tubes should be placed in a container covered with a wet towel in a sheltered, shaded location.

### 3.4.3 Number of uses of the impregnated papers

The efficacy of impregnated papers declines with the number of uses and the number of mosquitoes tested. This is especially true of the pyrethroid-impregnated papers. The current recommendation is that no insecticide-impregnated paper should be used more than six times, which is equivalent to exposing about 150 mosquitoes in a single tube. Previous versions of these procedures allowed greater reuse of the nonpyrethroid-impregnated papers (up to 20 times).

Between tests, papers should be kept in their original plastic box, sealed with tape and stored in a container or refrigerator at 4 °C or, if this is not possible, in a darkened cupboard at room temperature. If papers have been stored at 4 °C, they should be brought to room temperature before being used in an exposure test. Test papers should never be exposed to direct sunlight. Date of expiry of each batch is given on the box and should be strictly adhered to.

## 3.5 Mortality and adjustment calculations

A standard form that can be used for recording and reporting the results of bioassays, both mortality and knockdown rates, is included at Annex 2. The assessment of mortality (i.e. a count of the number of dead mosquitoes in both the exposure and the control tubes) is made at the end of the specified post-exposure period.

The mortality of the test sample is calculated by summing the number of dead mosquitoes across all exposure replicates and then expressing this as a percentage of the total number of exposed mosquitoes:

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \times 100$$

A similar calculation should be made in order to obtain a value for the control mortality. If the control mortality is ≥20%, the tests must be discarded. When control mortality is <20%, then the observed mortality must be corrected using Abbott's formula, as follows:

$$\text{Corrected mortality} = \frac{\text{(\% observed mortality – \% control mortality)}}{\text{(100 – \% control mortality)}} \times 100$$

If the control mortality is <5% (i.e. one dead mosquito out of 25), no correction of test results is necessary, whereas mortality of ≥5% requires correction.

When reporting mortality counts, the sample size should always be given, and preferably an estimate of the 95% confidence intervals.

## 3.6 Interpretation of results

### 3.6.1 Susceptibility tests with discriminating concentration

In light of new knowledge and the need for prompt action to counter the spread of resistance among vector populations, guidance on interpreting the results of the WHO susceptibility test was revised in 2013 *(9)*. The current recommendations for discriminating concentrations assays are as follows:

- A mortality in the range of 98–100% indicates susceptibility of the mosquitoes.
- A mortality of less than 98% is suggestive of the existence of resistance and further investigation is needed. If the observed mortality (corrected, if necessary) is between 90% and 97%, the presence of resistant genes in the vector population must be confirmed by additional tests with the same insecticide on the same population or on the progeny of any surviving mosquitoes (reared under insectary conditions), or by molecular assays for known resistance mechanisms. If at least two additional tests consistently show mortality below 98%, then resistance is confirmed.
- If mortality is less than 90%, confirmation of the existence of resistant genes in the test population with additional bioassays is not necessary, provided that at least 100 mosquitoes of each species were tested. It is then necessary to test additional samples at the 5× and 10× concentrations, as described in Section 3.6.2 below. Further investigation of the mechanisms and distribution of resistance should also be undertaken.

- When resistance is confirmed, remedial action is recommended whenever possible to manage insecticide resistance according to the GPIRM guidelines *(5)* and to ensure that the effectiveness of insecticides used for malaria vector control is preserved.

- In the event that unexpectedly high numbers of survivors are found following exposure to an insecticide that is expected to kill all test specimens, then the test papers concerned should be assayed against an *Anopheles* laboratory colony that is insecticide susceptible. Where there are no facilities to do this, papers should be sent to a regional laboratory or institution where these tests can be carried out to ensure that the quality of the papers is good.

### 3.6.2 Susceptibility tests with 5× and 10× discriminating concentrations

The current recommendations for interpretation of results from the intensity bioassays are as follows:

- Mortality (corrected, if necessary) of 98–100% at the 5× concentration indicates a low resistance intensity and it is not necessary to assay at the 10× concentration.

- Mortality (corrected, if necessary) of <98% at the 5× concentration indicates a moderate resistance intensity. It is recommended to assay further at the 10× concentration.

- Mortality in the range 98–100% at the 10× concentration confirms a moderate resistance intensity.

- Mortality (corrected, if necessary) of <98% at the 10× concentration indicates a high resistance intensity.

When resistance is confirmed at the 5× and especially at the 10× concentrations, operational failure is likely. Therefore, remedial action **MUST** be taken urgently to manage insecticide resistance *(5)* and to preserve the effectiveness of insecticides used for malaria vector control. Additionally, the distribution of resistance should be investigated to identify where resistance is most intensively expressed and where additional control measures should be prioritized if also indicated by epidemiological data.

The classification criteria given above (Sections 3.6.1 and 3.6.2) are recommended on the grounds that a survival of more than 2% is unlikely to be due to chance alone if all the test conditions are met.

Caution must be exercised when interpreting the results of individual bioassays, especially when using wild-caught females. Sampling techniques may influence the results; for example, indoor catches may be biased towards insects that may have already been exposed to insecticides and have survived (i.e. may include specimens that are more likely to be resistant). Mixed species samples may also produce inconclusive or misleading results because resistance gene frequency is likely to vary between species. Hence, it is important to identify test insects and to calculate susceptibility rates for each species separately.

## 3.7 Equipment and supplies

### 3.7.1 Procurement

Test kits and insecticide-impregnated papers are prepared in coordination with WHO by the Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia. The latest procedures and conditions for ordering standard test kits, impregnated papers and other related supplies are specified on the WHOPES website.[3] All items included as part of the test kits, including the impregnated papers, can be ordered separately, using the order form available on the website or via an online portal.[4] Full instructions for carrying out the susceptibility test are included as part of the kit, along with multiple copies of the recommended data recording forms (see Annex 1 and Annex 2).

### 3.7.2 Composition of the WHO test kit

The composition of the standard WHO test kit is given below, although additional tubes and metal clips may be separately ordered:

- Twelve plastic tubes (125 mm in length and 44 mm in diameter) with each tube fitted at one end with 16-mesh gauze:
    - four tubes marked with a **red** dot for use as exposure tubes (i.e. for exposing mosquitoes to insecticide-impregnated papers);
    - two tubes marked with a **yellow** dot for use as control tubes without insecticide (i.e. for exposing mosquitoes to the oil-treated control papers);
    - six tubes marked with a **green** dot for use as holding tubes for pretest sorting and post-exposure observation; and
    - six slide units each fitted with a screw cap on both sides and a 15 mm diameter filling hole.
- Forty sheets of clean white paper (12 × 15 cm) for lining the holding tubes.
- Twelve metal clips (six of steel and six of copper), to hold the paper in position against the walls of the tubes; the six steel clips are used with the six green-dotted holding tubes, and the six copper clips are used with the four red-dotted exposure tubes and the two yellow-dotted control tubes.
- Two glass aspirator tubes of 12 mm internal diameter, together with tubing 60 cm long and two mouthpieces.
- One roll of self-adhesive plastic tape.
- One instruction sheet.
- One report form.
- One log-probability paper.
- One label.

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3 http://www.who.int/whopes/resistance/en/

4 http:/www.inreskit.usm.my

### 3.7.3 Insecticide- and synergist-impregnated papers

The range of insecticides for which impregnated test papers are available has been expanded to include new active ingredients; intensity bioassay concentrations for pyrethroids, pirimiphos-methyl and bendiocarb; and 4% PBO (see Table 3.1). Insecticide-impregnated papers are packaged in plastic boxes; each box contains eight papers.

Users are encouraged to regularly consult the WHOPES website[5] for updates on the availability of test papers and other supplies. Papers impregnated with insecticides at serial concentrations, including the 5× and 10× concentrations (as listed in Table 3.1), are available upon request from the Universiti Sains Malaysia via the online portal.[6] The 5× and 10× papers are for use in situations where it is necessary to establish the intensity of resistance expression of a mosquito species or population to a given insecticide.

5 http:/www.who.int/whopes

6 http://www.inreskit.usm.my

# 4. FURTHER FIELD INVESTIGATIONS: SYNERGIST-INSECTICIDE BIOASSAYS AS A PROXY FOR METABOLIC RESISTANCE MECHANISMS

## 4.1 Use of synergists in insecticide susceptibility tests

As with the WHO susceptibility test, the synergist-insecticide bioassay is a direct response-to-exposure test. Specifically, it measures the effect of pre-exposure to a synergist on the expression of insecticide resistance. A synergist is not an insecticidal compound, but is recognized as a substrate by certain detoxifying enzymes within mosquitoes. This bioassay is used to assess the involvement of metabolic resistance mechanisms in the production of resistance phenotypes. The test procedure itself is summarized in Box 4.1. All issues pertaining to test conditions (including ambient conditions) are described in Section 3.4.

Synergists are available for certain metabolic detoxification enzyme groups including esterases, oxidases and glutathione *S*-transferases. For example, PBO can synergize the effects of pyrethroid insecticides by reducing or nullifying the detoxifying capabilities of enzymes, primarily monooxygenases. Partial or complete mitigation of the expression of a resistant phenotype implies that a monooxygenase-based detoxification system is primarily responsible for the resistance in the absence of PBO. Other synergist compounds are not as yet sufficiently validated for use in these synergist-insecticide assays.

BOX 4.1

**MEASURING THE EFFECT OF PRE-EXPOSURE TO THE SYNERGIST PBO ON THE EXPRESSION OF INSECTICIDE RESISTANCE IN ADULT MOSQUITOES: TEST PROCEDURE**

1. This experiment comprises four bioassay exposures: PBO only, insecticide only, PBO followed by insecticide, and solvent control.
2. Standard WHO test tubes are used for the bioassays and the transfer techniques described in Box 3.1 are used.
3. Four sheets of clean white paper (12 × 15 cm), rolled into a cylinder shape, are inserted into four holding tubes with green dots (one per tube) and fastened into position with a steel spring-wire clip. The tubes are attached to slides. Ensure that all tubes are appropriately labelled.
4. Five exposure tubes (four with red dots and one with a yellow dot) are prepared in much the same way. Two red-dotted exposure tubes are each lined with a sheet of insecticide-impregnated paper (insecticide discriminating concentration); the other two red-dotted tubes are each lined with 4% PBO-impregnated papers. The yellow-dotted control exposure tube is lined with oil-impregnated paper. Each paper is fastened into position with a copper spring-wire clip. Ensure that all tubes are appropriately labelled.
5. At least 100 adult females are selected from the mosquito sample in question.
6. The mosquito sample is divided into four sets of 20–25 mosquitoes, each of which is then aspirated into one of the four holding tubes.

7. The "PBO only" and "PBO followed by insecticide" test mosquitoes are transferred to the two 4% PBO exposure tubes for 1 hour. Mortality is recorded after 1 hour of exposure (there should be no mortality after 1 hour). The other samples ("insecticide only" and "solvent control") are left in their respective holding tubes.

8. The "PBO only" test mosquitoes are transferred back to their holding tube.

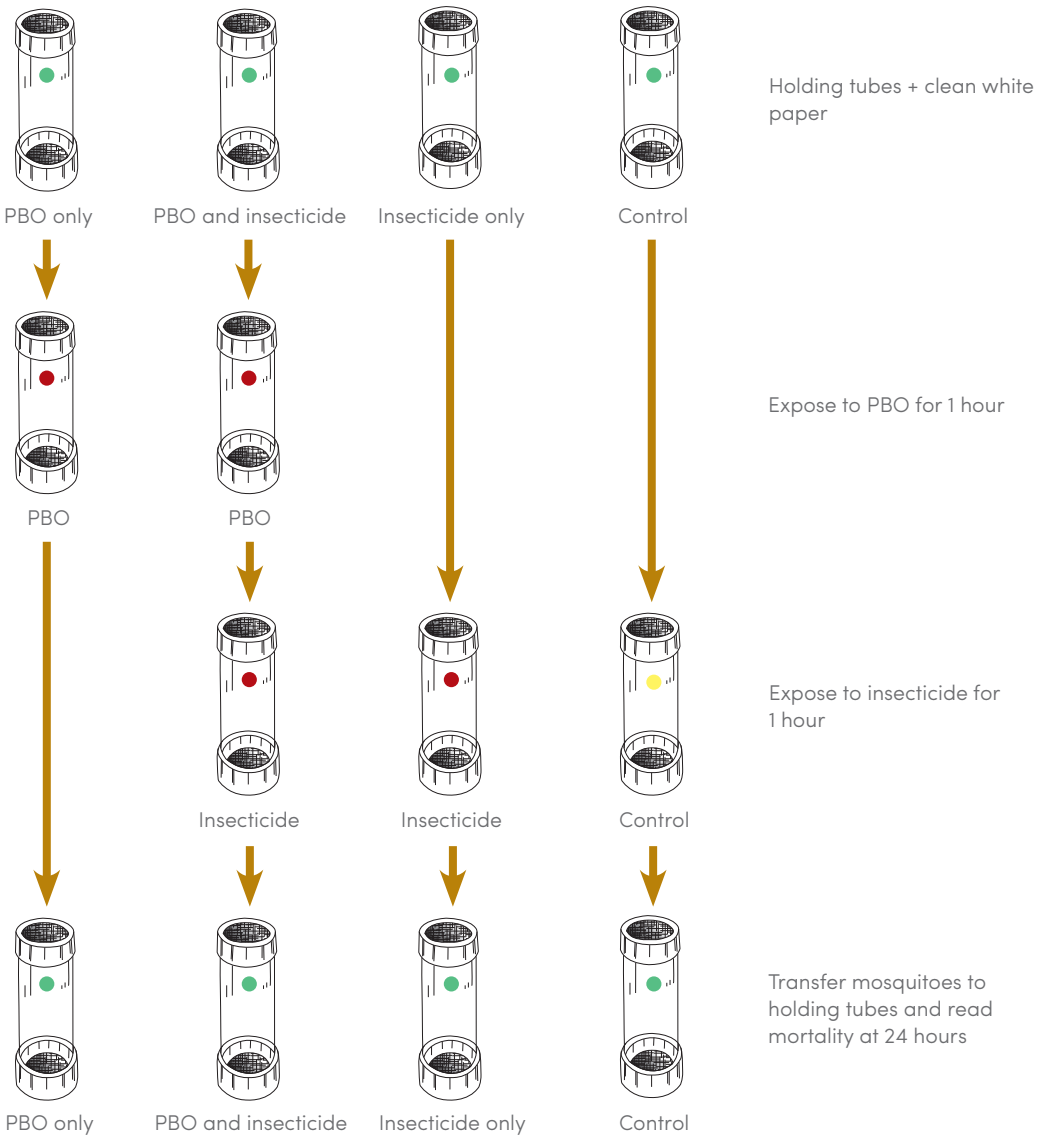
9. The "insecticide only" and "PBO followed by insecticide" test mosquitoes are simultaneously transferred to the two insecticide exposure tubes and exposed for 1 hour. At the same time, the "solvent control" test mosquitoes are transferred to the oil-impregnated paper control tube and exposed for 1 hour.

10. After the 1 hour exposure, the remaining three test mosquito samples ("insecticide only", "PBO followed by insecticide" and "solvent control") are transferred back to their respective holding tubes and mortality is recorded. A pad of cotton wool soaked in sugar water is placed on the mesh-screen end of each of the four holding tubes.

11. Mosquitoes are maintained in the holding tubes for 24 hours (the recovery period). During this time, it is important to keep the holding tubes in a shady, sheltered place free from extremes of temperature (an insectary is ideal). Temperature and humidity are recorded during the recovery period.

12. At the end of the recovery period (i.e. 24 hours post-exposure), the number of dead mosquitoes in each tube is counted and recorded according to the definitions given in Box 3.1.

13. This process is repeated three times. A schematic representation of the procedure is shown in Fig. 4.1.




PBO, piperonyl butoxide

FIG. 4.1
**Steps to perform the synergist-insecticide bioassay test**

## 4.2 Recording and reporting results from synergist-insecticide bioassay

### 4.2.1 Measuring mortality

The assessment of mortality (i.e. a count of the number of dead mosquitoes in the exposure and control tubes) is made 24 hours post-exposure. If, 24 hours after exposure, a mosquito is still able to fly, it should be counted as alive. If, however, a mosquito is "knocked down" (i.e. is moribund), it should be counted as dead. This can be justified on the grounds that, in the wild, a mosquito in this condition would probably be caught and eaten by predators or ants.

The mortality of the test and control samples is calculated by summing the number of dead mosquitoes and expressing this as a percentage of the total number of exposed mosquitoes:

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \times 100$$

If the "solvent control" mortality is > 20%, the tests must be discarded. When control mortality is ≤ 20%, then the observed mortalities for the "insecticide only" and "PBO followed by insecticide" exposures must be corrected using Abbott's formula, as follows:

$$\text{Corrected mortality} = \frac{\text{(\% observed mortality – \% control mortality)}}{\text{(100 – \% control mortality)}} \times 100$$

When reporting mortality counts, the sample size should always be given.

If mortality in the "PBO only" assay is > 10% (i.e. two or more dead mosquitoes per tube) then the entire test should be discarded on the grounds that the synergist papers are causing a significant degree of mortality.

### 4.2.2 Comparing samples exposed to synergist with those not exposed

Once corrections for mortalities based on the mortality with controls have been completed, the mortalities induced in the "PBO followed by insecticide" samples can be compared to those induced in the nonsynergized "insecticide only" samples.

## 4.3 Interpretation of synergist-insecticide test results

The current recommendations are as follows:

- If mean mortality in the "insecticide only" samples is ≥ 90%, the effect of PBO cannot be reliably assessed.
- If mean mortality in the "insecticide only" samples is < 90%, the effect of PBO can be interpreted according to the following criteria:
    - Complete restoration of susceptibility (mitigation of resistance) by pre-exposure to PBO (i.e. ≥ 98% mean mortality in the "PBO followed by insecticide" samples) implies that a monooxygenase-based resistance mechanism fully accounts for expression of the resistant phenotype in the test population.
    - Partial restoration of susceptibility by pre-exposure to PBO (i.e. mean mortality in the "PBO followed by insecticide" samples is greater than mean mortality in the "insecticide only" samples but < 98%) implies that a monooxygenase-based resistance mechanism only partially accounts for expression of the resistant phenotype and that other resistance mechanisms are likely to be present in the test population.

- No restoration of susceptibility by pre-exposure to PBO (mean mortality in the "PBO followed by insecticide" samples is equal to or lower than mean mortality in the "insecticide only" samples) implies that the resistance phenotype detected is not based on monooxgenase-mediated detoxification.

## 4.4 Equipment and supplies

Test kits, insecticide-impregnated papers and 4% PBO-impregnated papers are prepared on behalf of WHO by the Universiti Sains Malaysia, as previously described (see Section 3.7).

Copies of the recommended data recording form are included in the kits and are at Annex 3.

# 5. THE CDC BOTTLE BIOASSAY FOR ADULT MOSQUITOES

A detailed description of the CDC bottle bioassay, including the methodology, was published by the CDC in 2010 *(16)*. That publication is periodically updated through additional material (e.g. the intensity bioassay protocol) being made available on the website, which includes an instructional video of the guidelines.[7] A printable version of the guidelines with photographs is also available *(25)*.

## 5.1 Bottle bioassay with discriminating concentrations and times

The CDC bottle bioassay provides a complementary method for detecting insecticide resistance in malaria vector populations and is widely used for routine, day-to-day monitoring of resistance in mosquito populations. Whereas the WHO bioassay measures mortality rates in mosquitoes exposed to a discriminating concentration of insecticide for a fixed period of time, the CDC bottle bioassay measures a discriminating length of time required to incapacitate susceptible mosquitoes using a predetermined concentration of insecticide.

As with the WHO bioassay, the CDC bottle bioassay can be performed on adult females collected from the field or on those reared in an insectary from larval collections. Also, the bottle bioassay has been standardized by determining discriminating concentrations and exposure times for individual insecticides and for each main vector species using populations known to be susceptible. Testing can therefore also be done at the discriminating concentration and time only (Table 5.1). The test involves recording the number of mosquitoes incapacitated or knocked down after the discriminating exposure time (i.e. the time that reliably incapacitated 100% of the original test population).

## 5.2 Bottle bioassay with intensity concentrations

Recently, the bottle bioassay has been enhanced through development of an intensity bioassay in this format. A series of multiples of the discriminating concentration (1×, 2×, 5× and 10×) gives similar information to that obtained through calculation of resistance ratios. However, through the use of concentration multiples, valuable information can be gained with far fewer mosquitoes, making the intensity bioassay approach a rapid discriminating test for assessing the potential significance for control failure. If mosquitoes survive at a higher concentration multiple, this alerts a resistance surveillance team to a site where resistance may be compromising control, and which therefore requires further analysis.

## 5.3 Advantages and disadvantages of bottle bioassay

If the CDC bottle bioassay is to be used for routine insecticide susceptibility surveillance, the following conditions should be noted:

- the procedures detailed in the CDC guidelines should be strictly adhered to; in particular, those procedures relating to the use of the recommended insecticide solvents (ethanol or acetone) and the bottle treatment protocols;

7 http://www.cdc.gov/parasites/education_training/lab/bottlebioassay.html

- insecticide exposure times and concentrations (listed in Table 5.1) should be adhered to; and
- storage times and bottle-washing processes must be strictly adhered to.

Advantages of using the CDC bottle bioassay method include the following:

- if users choose to prepare their own bottles, they can avoid the use of pre-prepared test kits and insecticide-impregnated papers, allowing greater flexibility in the type and concentration of insecticide that can be evaluated;
- the manual includes a protocol for using formulated insecticides in the bioassay analysis, although this requires a careful interpretation of results by the user;
- the procedure is relatively simple and quick (e.g. no requirement for a 24-hour or longer holding period); and
- the procedure can be performed with various synergists.

Disadvantages of using the CDC bottle bioassay method include the following:

- possible issues with control assurance – the responsibility for correctly preparing the glass bottles rests with the end user but, for example, different laboratories could differ in the way they prepare the equipment before and after testing;
- the need for entomologists to wear personal protective equipment to treat the bottles;
- the need to transport glass bottles in the field, particularly over extended periods when access to laboratories is not possible;
- the requirement to transfer to a clean cage before separating dead and live mosquitoes for subsequent identification of species and investigation of resistance mechanisms; and
- the need to thoroughly clean all bottles between uses – random checking of washed bottles using susceptible mosquitoes is essential.

Both the WHO and the CDC method can reliably identify insecticide resistance where it occurs. However, although both assays report either percentage mortalities or time to incapacitation of mosquitoes, the results obtained from the CDC bottle bioassay are not directly comparable with those obtained from the WHO susceptibility tube test. This is because the CDC method focuses on the proportion of mosquitoes incapacitated, whereas the WHO bioassay is concerned with mortality.

TABLE 5.1

**CDC bottle bioassay insecticide discriminating concentrations and discriminating times (in minutes) for *Anopheles* and *Aedes* mosquitoes**

| INSECTICIDE | INSECTICIDE CONCENTRATION PER SPECIES (MICROGRAM ACTIVE INGREDIENT PER 250 ML WHEATON BOTTLE) | | DISCRIMINATING TIME (MINUTES) |
|---|---|---|---|
| | *ANOPHELES* | *AEDES* | |
| Bendiocarb | 12.5 | 12.5 | 30 |
| Cyfluthrin | 12.5 | 10 | 30 |
| Cypermethrin | 12.5 | 10 | 30 |
| Deltamethrin | 12.5 | 10 | 30 |
| Lambdacyhalothrin | 12.5 | 10 | 30 |
| Permethrin | 21.5 | 15 | 30 |
| DDT | 100 | 75 | 45 |
| Malathion | 50 | 50 | 30 |
| Fenitrothion | 50 | 50 | 30 |
| Pirimiphos-methyl | 20 | – | 30 |

DDT, dichlorodiphenyltrichloroethane

# 6. FURTHER LABORATORY INVESTIGATIONS: IDENTIFICATION OF RESISTANCE MECHANISMS

As noted in Section 4, if survivors are found following use of the discriminating concentrations (> 2%), it may be beneficial to conduct further tests, to determine the underlying genetic mechanisms responsible for the observed resistance. This information is likely to prove useful in longer term vector-control planning, where it will both assist in assessing the likelihood of cross-resistance between compounds in insecticide classes and at the same time provide valuable information about the potential for spread of resistance in vector populations. For instance, if evidence of the presence of *kdr* mutations (which confer resistance against the pyrethroids) was found in a given vector population, it is likely that the same population would also be resistant to DDT.

As understanding of the mechanisms of insecticide resistance in mosquitoes and other insect vectors has evolved, significant progress has been made in developing new tests for the detection of resistance mechanisms. A range of biochemical enzyme assay techniques that detect the presence of metabolic resistance mechanisms have been available for several decades. They are now supplemented by several molecular assays that can be used to test for target-site mutations (e.g. *kdr* for pyrethroids and DDT, *Ace-1* for the organophosphates and carbamates, and *rdl* for fipronil and dieldrin) and, increasingly, for metabolic resistance mechanisms (e.g. glutathione *S*-transferases). In settings where resources and facilities are limited, help with analysing representative samples should be sought from external institutions. Similarly, advice on specimen preservation should be sought from partner institutions.

Detailed descriptions of the recommended techniques and methods for biochemical and molecular analysis are beyond the scope of these guidelines. For this type of information, users are advised to consult other sources, such as the *Methods in Anopheles research manual (25)* or other published reports *(27)*.

# 7. DATA MANAGEMENT AND USE

Ideally, data form the basis for public health action. Effective management, rapid sharing and timely appraisal of data are crucial to inform malaria vector-control strategies and implementation. Information generated from insecticide resistance monitoring is a key component for decision-making on insecticidal interventions, in conjunction with epidemiological and other available data.

## 7.1 Management and sharing of data

Responsibility for the collection, quality check, collation, analysis and reporting of data ultimately lies with the national vector-borne disease control programmes. This ensures available resources are used effectively to generate the information needed to guide timely decision-making.

If a country has no national database for entomological information including insecticide resistance, such a database should be established. Potential linkages with existing data management functions and tools (e.g. a health management information system) should also be explored. Where there is limited capacity in the NMCP, the data management function may be performed by another institution, such as a research institution, on behalf of the programme. The procedures for submitting, managing and reporting of data should be well documented. The process, frequency and format for making data available to the NMCP should be clearly established among partner institutions.

All partners involved in the collection of relevant entomological data should be encouraged to provide these data for input into the national database in a timely manner. Availability of standardized templates aligned with the national database will streamline the data collation process and ensure appropriate reporting. Ideally, results of insecticide susceptibility and mechanisms testing should be made available to the NMCP within 3 months of their collection.

Data on bioassays, including intensity assays where done, and resistance mechanism tests should also be submitted to WHO for inclusion in the aggregated global database that has been established since 2014. The global database builds on regional databases, and includes information provided annually by NMCPs as well as that derived from publications and regional networks. A standard insecticide resistance data template is available to aid the process of reporting to WHO. The comprehensive information is evaluated to better understand the regional and global resistance situation, and is used to guide monitoring and vector-control policy, as outlined in the GPIRM.

The utility of the global databases is highly dependent on collation and reporting of data by malaria-endemic countries, underscoring the importance of data quality, and of timely and complete data sharing at national level.

## 7.2 Use of data in decision-making

National insecticide resistance monitoring data should be reviewed and appraised periodically by a convened group of technical experts in order to generate informed decisions on vector control. Since the NMCPs have the mandate and responsibility

for malaria vector control at country level, such programmes should lead the establishment and functioning of this technical body. This body should be responsible for the coordination of national activities on insecticide resistance monitoring and management, to ensure appropriate prioritization and use of resources, and to provide a mechanism for efficient decision-making.

The NMCP should decide on the most appropriate composition of the group. A partner-mapping exercise should be an initial step in the process of identifying institutions and individuals that will participate in the national decision-making body, or that can provide relevant data (e.g. on insecticide susceptibility) and resources to support decision-making. It is imperative to involve partners in this process, to ensure that any changes in vector-control policy or insecticide resistance management and monitoring plans reflect the current situation, and can be funded and implemented effectively. Strong intersectoral involvement is recommended, including representatives of ministries of health, agriculture and environment, and technical experts from WHO and academic institutions, as well as other appropriate partners such as donors and nongovernmental organizations. Participation by the national regulatory authority is crucial to enable informed selection of appropriate insecticidal interventions.

The priority for a decision-making body is then to review the existing vector-control strategy and practice with regards to technical updates on insecticide resistance, and make appropriate adjustments to align with the GPIRM and subsequent guidance from WHO. Further guidance and examples of the structure of the technical and decision-making body are contained in the GPIRM *(5)*. The documented process undertaken in Zambia for operational planning for insecticide resistance management is also informative *(28)*.

# 8. ADDITIONAL RECOMMENDATIONS

A number of general recommendations regarding insecticide resistance monitoring merit a mention:

- As part of GPIRM implementation *(5)*, malaria or vector-borne disease programmes should develop insecticide resistance management plans detailing priority interventions, selection of sentinel sites, and frequency of monitoring of vectors and insecticide susceptibility. These plans should include data management to inform programmatic decisions.

- Efficient resistance-monitoring programmes are dependent on adequately trained personnel and sufficient infrastructure. This is still a major challenge in most countries, and is being addressed by a number of institutions and implementing partners. Training needs at country level should be reassessed and addressed.

- Simple, rapid throughput PCR-based molecular tests have been developed in recent years and are currently being used to detect the presence of *kdr* mutations. For example, such methods have been used to monitor the frequency of the *kdr* mutations in *An. gambiae* in western parts of Africa as a proxy for estimating the presence of DDT or pyrethroid resistance. This is not a generally recommended practice, and the WHO susceptibility tests (or the CDC bottle bioassays) should always be carried out in addition to the molecular assays. Of course, knowledge of the mechanisms involved in resistance is always advantageous because cross-resistance between classes of insecticides can be inferred and bioassays can then be used to test for resistant phenotypes. This type of information is useful for planning and assessing success or failure of resistance management strategies.

- Where laboratory facilities are available, resistance data can be linked to parasite infection data by processing the same wild adult mosquitoes used in bioassays for sporozoite detection. Specimens that test positive in an initial enzyme-linked immunosorbent assay (ELISA) should be confirmed by a second (ELISA) that has been heated *(29)* or by PCR. This is relevant in terms of the impact of resistance on malaria transmission.

- For novel insecticides that are not acting primarily through lethal effects but that disturb the insect physiology (e.g. blood-feeding behaviour, or reduction in fecundity or fertility), other procedures for testing resistance will need to be developed.

- In situations where few specimens are available, strategic testing against a single class 1 pyrethroid (e.g. permethrin) and a single class 2 pyrethroid (e.g. deltamethrin) should be sufficient. Vector-control programmes may consider initial testing of resistance against only permethrin and deltamethrin among the pyrethroids, bendiocarb among the carbamates, either malathion or pirimiphos-methyl among the organophosphates, and DDT from the organochlorines.

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## ANNEX 1. FORM FOR RECORDING INFORMATION ON MOSQUITO COLLECTIONS AND TEST CONDITIONS

Village code ☐☐☐ Test number ☐☐☐☐ Date (dd-mm-yy) ☐☐/☐☐/ ☐☐

Investigator name: ..... Code of investigator ☐☐☐

**Area information**

Country: ..... Province: .....

District: .....Commune: .....Village: .....

GPS position UTM_X ☐☐☐.☐☐☐ UTM_Y ☐☐☐.☐☐☐

**Sample information**

Species tested: .....Species control: .....

Sex: ..... Age (days): ..... (only if known: colony & F1)

**Collection method**

Human landing indoor ☐ Resting night indoor ☐ Resting morning indoor ☐

Cattle collect ☐ Human landing outdoor ☐ Resting night outdoor ☐

Other: specify ..... Larval collection ☐ Progeny F1 ☐

Colony ☐ Name of colony strain: .....

**Physiological stage**

Non-blood fed ☐ Blood fed ☐ Semi-gravid ☐ Gravid ☐

**Test insecticide information**

Insecticide tested: ..... Date of expiry: ☐☐/☐☐/☐☐

Impregnated papers prepared by: ..... Date box first open: ☐☐/☐☐/☐☐

Concentration (1x/5x/10x): .....

Number of times this paper is used: ☐☐

Storage conditions: Room temperature ☐ Refrigerated ☐

**Test conditions**

| | Exposure period: Start | End test |
|---|---|---|
| Temperature °C | ☐☐.☐ | ☐☐.☐ |
| Relative humidity (%) | ☐☐ | ☐☐ |

# ANNEX 2. FORM FOR RECORDING RESULTS OF SUSCEPTIBILITY TESTS USING DISCRIMINATING, 5× AND 10× CONCENTRATIONS

| | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Control 1 | Control 2 |
|---|---|---|---|---|---|---|
| No. exposed | | | | | | |

**Number of knocked down mosquitoes after exposure for 60 minutes (120 minutes for fenitrothion)**

| | Replicate 1 | | Replicate 2 | | Replicate 3 | | Replicate 4 | | Control 1 | | Control 2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Time | No. | Time | No. | Time | No. | Time | No. | Time | No. | Time | No. |
| Start | | | | | | | | | | | | |
| 60' | | | | | | | | | | | | |

**Number of dead and number of alive mosquitoes* at the end of holding period (24 hours)**

| | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 4 | Control 1 | Control 2 |
|---|---|---|---|---|---|---|
| No. dead | | | | | | |
| No. alive | | | | | | |

* These counts need to be by species, once dead and alive mosquitoes have been examined for taxonomic determination.

**To be completed by a supervisor at the end of the test**

Code of supervisor ☐☐☐

Comments .....

.....

.....

I confirm that the form is complete.

Date: ☐☐/☐☐/☐☐

Name .....

Signature .....

**To be completed by data entry clerks during data entry**

Data entry clerk 1

Date ☐☐/☐☐/☐☐

Signature .....

Data entry clerk 2

Date ☐☐/☐☐/☐☐

## ANNEX 3. FORM FOR RECORDING RESULTS OF SYNERGIST-INSECTICIDE (4% PBO) ASSAYS

**Number of knocked down mosquitoes after exposure by treatment for each replicate**

| **Replicate 1** | Insecticide only | | PBO only | | Insecticide + PBO | | Solvent control | |
|---|---|---|---|---|---|---|---|---|
| | Time | No. | Time | No. | Time | No. | Time | No. |
| Start | | | | | | | | |
| End | | | | | | | | |
| **Replicate 2** | Insecticide only | | PBO only | | Insecticide + PBO | | Solvent control | |
| | Time | No. | Time | No. | Time | No. | Time | No. |
| Start | | | | | | | | |
| End | | | | | | | | |
| **Replicate 3** | Insecticide only | | PBO only | | Insecticide + PBO | | Solvent control | |
| | Time | No. | Time | No. | Time | No. | Time | No. |
| Start | | | | | | | | |
| End | | | | | | | | |

**Number of dead and number of alive mosquitoes at the end of the holding period (24 hours) by treatment for each replicate**

| **Replicate 1** | Insecticide only | PBO only | Insecticide + PBO | Solvent control |
|---|---|---|---|---|
| No. dead | | | | |
| No. alive | | | | |
| **Replicate 2** | Insecticide only | PBO only | Insecticide + PBO | Solvent control |
| No. dead | | | | |
| No. alive | | | | |
| **Replicate 3** | Insecticide only | PBO only | Insecticide + PBO | Solvent control |
| No. dead | | | | |
| No. alive | | | | |

## Summary table showing mean percentage mortalities recorded for samples of *Anopheles* adult females following exposure to either insecticide only, PBO (4%), insecticide + PBO or untreated solvent control papers

| Treatment | No of replicates | Sample size (N) | Mean % mortality 24 hours post exposure |
|---|---|---|---|
| Insecticide only | | | |
| PBO only | | | |
| Insecticide + PBO | | | |
| Solvent control | | | |

PBO, piperonyl butoxide

**To be completed by a supervisor at the end of the test**

Code of supervisor ☐☐☐

Comments .....

.....

.....

I confirm that the form is complete.

Date: ☐☐/☐☐/☐☐

Name .....

Signature .....

**To be completed by data entry clerks during data entry**

Data entry clerk 1

Date ☐☐/☐☐/☐☐

Signature .....

Data entry clerk 2

Date ☐☐/☐☐/☐☐

## ANNEX 4. DISCRIMINATING CONCENTRATIONS AND EXPOSURE TIME OF INSECTICIDES COMMONLY USED FOR *AEDES* MOSQUITOES

| Insecticide class | Insecticide | Discriminating concentrations (%) | Exposure period (hours) |
|---|---|---|---|
| Pyrethroids | Alpha-cypermethrin | 0.03[a] | 1 |
| | Cyfluthrin | 0.15[b] | 1 |
| | Deltamethrin | 0.03[a] | 1 |
| | Etofenprox | 0.5[b] | 1 |
| | Lambdcyhalothrin | 0.03 | 1 |
| | Permethrin | 0.25 | 1 |
| Organophosphate | Fenitrothion | 1 | 1 |
| | Malathion | 0.8 | 1 |
| | Pirimiphos methyl | 0.21[b] | 1 |

[a] Tentative

[b] Determined for *Anopheles* mosquitoes, tentative for *Aedes*.